

Supplementary Materials and methods

Multimodule analysis

Bulk-tumor RNA-seq (n = 255) was utilized to stratify the FU-iCCA cohort. Then, WES (n = 249) and proteomics (n = 208) data elucidated the trans-omics connections and underlying molecular mechanisms. Meanwhile, H&E staining (n = 177), multiplex immunostaining (n = 188), and scRNA-seq (n = 10) were used to validate the immune features. The details of WES, RNA-seq, and proteomics analyses were conducted as described in our recent publication¹ and also briefly summarized as follows:

DNA Extraction and WES analysis

Genomic DNA was extracted from tumor and non-tumor liver tissues using QIAamp Fast DNA tissue kit (QIAGEN) according to manufacturer's protocol. DNA was quantified by the Qubit 3.0 (Invitrogen) and NanoDrop 2000 (Thermo Scientific). WES libraries were prepared and captured using the QuarPrep EZ DNA Library Kit and QuarHyb Reagent kit (DynastyGene Biotechnologies) following the manufacturer's instructions. The DNA library with 150 bp paired-end reads was sequenced with Illumina NovaSeq 6000 System. WES was conducted with a mean coverage depth of 205X (range: 90-300X) for tumor samples and 97X (range: 47-162X) for adjacent non-tumor liver samples. The exome sequencing data was first aligned to GRCh37/hg19 sequence reference using BWA MEM v 0.7.10 (<http://bio-bwa.sourceforge.net/>). After alignment, the GATK best practice workflow was applied on mapping results, including marking duplicates with picard v2.22.8

(<https://broadinstitute.github.io/picard/>) and base quality recalibration with GATK BaseRecalibrator v4.1.7.0 (<https://gatk.broadinstitute.org/hc/en-us>). Following the GATK guidance on tumor-normal matching samples, short somatic variants were called and filtered using GATK Mutect2 and other components. These variants were annotated using SnpEff v4.3T (<https://pcingola.github.io/SnpEff/>) and Annovar v2019-10-24 (<https://doc-openbio.readthedocs.io/projects/annovar/en/latest/>). variants filtering procedure, including the Catalogue Of Somatic Mutations In Cancer (COSMIC) (v91, 2020-04-07) (<https://cancer.sanger.ac.uk/cosmic>), the 1000 Genomes (v5b, 2015-08-24), NHLBI Exome Sequencing Project (ESP6500) (v2, 2014-12-22) (<https://evs.gs.washington.edu/EVS/>) and the Genome Aggregation Database (gnomAD) (v2.1.1 2019-04-09) (<https://gnomad.broadinstitute.org/>). To obtain high quality somatic variants, stringent downstream filters were used.

RNA Extraction and RNAseq analysis

Total RNA was extracted and purified from fresh frozen tissues using the Whole RNA extraction kit (TIAN GEN). RNA integrity was measured on an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA with enough amount and good quality were used to prepare the transcriptome library. The cDNA synthesis, end-repair, A-base addition, and ligation of the Illumina index adapters were performed according to QuarPrep RNA Library Kit (DynastyGene Biotechnologies). Library quality was measured on an Agilent 2100 Bioanalyzer for product size and concentration. Paired-end libraries were sequenced by an Illumina NovaSeq 6000 System, with a sequence coverage of 139 million paired reads. RNA-seq data analysis identified 20,173 protein-coding genes with an average of 14,255 genes per sample, covering

the majority of the genes in proteomics. Sequence data were removed adaptors and low-quality reads using the Trimmomaticv 0.36. The cleaned sequence data were aligned to human reference genome (UCSC hg19 assembly) by STAR2(2.7.3a) in two-pass mode (with parameters: --chimSegmentMin 30 --chimJunctionOverhangMin 10 --alignSJDBoverhangMin 10 --alignIntronMax 200000 --alignSJstitchMismatchNmax 5 -1 5 5). The cleaned sequence reads were used for further qualification of gene expression. transcripts per million (TPM) values of each gene and transcript were calculated by Salmon with parameters of --seqBias --gcBias --posBias.

Proteomic Sample Preparation and Proteomic Analysis

Samples for MS were prospectively collected and 500 µL SDS lysis buffer was added into the powdered tissues for protein extraction, and sonicated at 20% amplitude for the total working time of 2 min with 5 s on and 5 s off (JY92-IIDN, Ningblio Scientz Biotechnology Co., LTD, China). The proteins were denatured at 95 °C for 5 min. Lysates were centrifuged at 12,000 g for 10 min to remove the insoluble debris and retain the supernatant for proteomic experiment. The tryptophan-based fluorescence quantification method was used to determine the protein concentration (Thakur et al., 2011). The equivalent of protein was digested by filter-aided sample preparation (FASP) procedure with 10 kDa centrifugal filter tubes (Millipore) and centrifuged at 12,000 g at 22 °C. Concentration for eluted peptides was determined by BCA protein quantification kit. 400 µg peptides were dried by vacuum freeze-drying for the following experiment. The mixed samples proteins for the 'internal reference' were

also digested by FASP as well as the other protein samples. 41 μL x 2 anhydrous acetonitrile was added into two sets TMT reagents (0.8 mg) and mixed with 400 μg peptides (dissolved in 200 μL 100 mMTEAB) per channel. The samples were incubated for 1 hr at room temperature, and then quenched the labeling reaction by adding 16 μL 5% hydroxylamine into the samples and was incubated for 15 min at room temperature. The pooled samples of the labeled peptides in one set were dried by vacuum freeze-drying for the following desalting experiment through the C18 solid-phase extraction (3M Empore). 0.1% formic acid was used to resolve the peptides and 1 μg resolved peptides for proteomic analysis were separated on an Easy nLC 1000 UHPLC system (Thermo Fisher Scientific) with a 120 min LC gradient at 300 nL/min (Buffer A: 0.1% formic acid in water; Buffer B: 0.1% formic acid in acetonitrile) of a home-made 75 mm x200 mm diameter C18 column (ReproSil-Pur C18-AQ, 3.0 μm resin (Dr. Maisch GmbH, Germany)). The column was heated to 50°C using an in-house column heater and the gradient was set as 2%–5% B in 1 min; 5%–25% B in 94 min; 25%–40% B in 15 min; 40%–60% B in 3 min; 60%–100% B in 1 min; 100% B in 6 min. The spray voltage of the Q Exactive HF-X mass spectrometer was set at 1,800 V in positive ion mode and the ion transfer tube temperature was set at 320 °C. Xcalibur software was used for data-dependent acquisition. The 24 benchmark fractions were analyzed with a 90 min LC gradient and the gradient was set as 2%–5% B in 1 min; 5%–25% B in 67 min; 25%–40% B in 13 min; 40%–60% B in 3 min; 60%–100% in 1 min; 100% B in 5 min. The parameters of Q Exactive HF-X mass spectrometer were set the same as iCCA proteomic samples.

Download of TCGA data and calculation of immune scores

Updated FPKM gene expression data, clinical data and sample information in TCGA database were obtained from the Genomic Data Commons (<https://portal.gdc.cancer.gov/>) using the R package TCGAbiolinks². Only primary solid tumor samples were enrolled in the analysis of immune score, which was calculated use R package ESTIMATE³.

Microenvironment-related gene expression analysis and clustering

Gene expression data was log-transformed using $\log_2(\text{FPKM}+1)$. We first selected immune-cell-related markers of 21 immune cells and 2 stromal cells from the xCell database⁴. Then correlations between expression level of all genes and prognosis were calculated. For each gene, patients were separated into two subgroups by the mean value of the gene expression level, and the Cox proportional hazards regression model was used to calculate *P* value between subgroups and prognosis using R package survival. 4,782 prognosis-related genes were filtered with $P < 0.05$. After intersected with immune-cell-related markers, 170 genes were selected as pivotal microenvironment genes. Unsupervised hierarchical clustering method was used to classify 255 iCCA patients into different subgroups with clustering method 'ward.D2' and distance 'manhattan'. To identify the optimal cluster number, the ConsensusClusterPlus package and silhouette analysis in cluster package was used to assess clustering stability. Finally, three subgroups were identified (IG1, IG2, IG3).

Calculation of differentially expressed genes/proteins and functional enrichment analysis

The 170 pivotal microenvironment genes could be divided into two gene subgroups, IG1 enrichment and IG3 enrichment. We performed gene ontology (GO) enrichment analysis to identify the enrichment of functional pathways of two gene subgroups using the R package clusterProfiler. Differentially expressed genes and proteins between different conditions were calculated using R package limma. Genes with adjusted P value < 0.05 and fold change > 1.5 ($\log_2FC > 0.58$) were set as significantly up-regulated, whereas with adjusted P value < 0.05 and fold change < 0.67 ($\log_2FC < -0.58$) were set as significantly down-regulated. Proteins with adjusted P value < 0.05 and fold change > 1.25 ($\log_2FC > 0.32$) were set as significantly up-regulated, whereas with adjusted P value < 0.05 and fold change < 0.8 ($\log_2FC < -0.32$) were set as significantly down-regulated. Another functional enrichment analysis method, Gene Set Enrichment Analysis (GSEA), was also used to analysis differentially regulated pathways via R package clusterProfiler. Gene set enrolled in the GSEA analysis were download from the Molecular Signatures Database (MSigDB, v7.1) of the Broad Institute⁵. HALLMARK gene sets (H) and MSigDB curated gene sets of KEGG (C2), GO (C5), REACTOME (C2) were used to perform GSEA in a 1,000-gene-set with a two-sided permutation⁶.

Calculation of immune-related and tumor-related scores

For the calculation enrichment score of different types of immune cells in each sample, we used single sample GSEA (ssGSEA) via GSVA package, with gene-sets of these immune cells downloaded from xCell database⁴. For the calculation of immune score (represents the infiltration of immune cells in tumor tissue) and stromal score (captures the presence of stroma in tumor tissue), R package ESTIMATE was used to predict the presence of infiltrating stromal/immune cells in tumor tissues using gene expression matrix³. The immunophenoscore of each patient was calculated using the algorithm and R script provided by Pornpimol Charoentong et al⁷. The mutation structure of KRAS were plotted using ProteinPaint.

Neoantigen predictions

Neoantigens were predicted by NetMHC (v4.0) and NetMHCpan (v4.1). The candidate neoantigens were filtered follow the criteria: 1) predicting as binders ($IC_{50} < 500$ nM in NetMHC or $Score_{EL} > 0.1$ in NetMHCpan). 2) neoantigen-derived genes are expressed in bulk RNA-seq data.

HLA genotyping and HLA-LOH prediction

POLYSOLVER was applied to investigate HLA gene genotypes. LOHHLA⁸ was used to predict the LOH events for HLA genes based on the POLYSOLVER results. HLA LOH events were identified using the parameter $P < 0.001$. The coverage depth on HLA gene locations of tumor and normal samples of each patient were manually checked to verify the HLA LOH events.

Immunoediting analysis

We followed the method developed by Rooney et al.^{9, 10} to analyze immunoediting in each patient. TCGA-HCC data was used as the driven null model. Referencing the published algorithm⁸, 1,827 synonymous SNVs and 4,927 nonsynonymous SNVs were enrolled in the analysis. To estimate immunoediting, 3,390 mutational spectra were considered.

Somatic Copy Number Alteration (SCNA) analysis

For each patient, SCNAs were inferred by CNVkit¹¹ with default parameters and identified by Genomic Identification of Significant Targets in Cancer (GISTIC, version 2.0)¹². The significantly gained or lost SCNA regions were determined by the default parameters in GISTIC and log₂ ratio cut-off of ± 0.8 was used to define SCNA amplification and deletion. The value of SCNA burden was calculated as the percentage of genes with amplification, gain, loss or deletion in each patient.

TCR and BCR analyses

Mapped RNA-sequencing reads were used to allelotype each patient by profiling TCR and BCR sequences with MiXCR as previously described¹³.

Multiplexed immunostaining of Tissue MicroArray (TMA). The TMA of the FU-iCCA cohort was used to validate our immune subgroups. Multiplex staining of

CD1a (ab108309, Abcam), CD3 (ab135372, Abcam), CD8 (ab93278, Abcam), CD15 (ab135377, Abcam), CD66b (ab197678, Abcam), CD20 (ab78237, Abcam), CK19 (ab52625, Abcam), PDPN (ab236529, Abcam), and CD31(ab76533, Abcam) was performed by the Vectra Automated Quantitative Pathology Imaging and Analysis platform through multispectral imaging system and inForm™ image analysis software (PerkinElmer). Following the manufacturer's instruction (PerkinElmer, Opal® Kit), we scanned the whole field of each sample by using the PerkinElmer Vectra3® platform and quantified the results by using PerkinElmer Vectra3® platform as is described before¹⁴. All quantifications were evaluated blinded to patient clinical outcomes.

Pathological examination. The presence of intra-tumoral TLSs was assessed morphologically on H&E staining slides, using a previously published scale¹⁵. Briefly, TLSs were classified as: i) Aggregates (Agg): vague, ill-defined clusters of lymphocytes; ii) Primary follicles (FL-I): round-shaped clusters of lymphocytes without germinal center formation and iii) Secondary follicles (FL-II): follicles with germinal center formation. Cases were also further scored according to TLS maturation stages: i) Agg iCCA: tumors with only Agg and no FL-I or FL-II; ii) FL-I iCCA: tumors with at least FL-I, with or without Agg and without FL-II and iii) FL-II iCCA: tumors with at least 1 FLII regardless of the presence of Agg and FL-I.

Pathology TILs were estimated from H&E staining slides using international established guidelines, using a previously published scale¹⁶. Briefly, the relative

proportion of stromal area to tumor area was determined from the pathology slide of a given tumor region. TILs were reported for the stromal compartment (= per cent stromal TILs).

Mouse model construction

Six weeks old female FVB/N mice were ordered from Shanghai Branch of Beijing Vital River Laboratory Animal Technologies Co. Ltd. maintained under SPF housing with a maximum of five mice per cage. The experiments were performed following the institutional guidelines strictly and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Shanghai Branch of Beijing Vital River Laboratory Animal Technologies Co. Ltd. (2017-0014). A sterile 0.9% NaCl solution/plasmid mix was prepared containing DNA. We prepared 20 µg of pT3-EF1α-HA-myr-Akt (Addgene, 31789), 30 µg of pT3-EF1α-YAP^{S127A} (Addgene, 86497), 10 µg of pT3-EF1α-KRAS^{WT} or 10 µg of pT3-EF1α-KRAS^{G12D}, and a 10:1 ratio of transposon to SB-luc transposase–encoding plasmid dissolved in 2 mL of 0.9% NaCl solution. Mice were injected with the solution into the lateral tail vein with a total volume corresponding to 10% of body weight in 6 to 8 seconds. Vectors for hydrodynamic delivery were produced using the QIAGEN plasmid PlusMega kit. Equivalent DNA concentration between different batches of DNA was confirmed to ensure reproducibility among experiments

Single cell RNA sequencing of mouse iCCA models

Tumors from AY (n = 6) and AYK (n = 6) iCCA mouse models were dissected using the Miltenyi Mouse Tumor Dissociation Kit and gentleMACS Octo-Dissociator (Miltenyi) following manufacturer's instructions. After filtering through a 70-mm filter, tumors were selected and droplet-based isolation of single cells was performed. Then, the cells were resuspended in 500 μ L sample buffer and placed on ice and cell viability was tested using a BD Rhapsody Scanner instrument. Calcein AM (Invitrogen) and Drap7 (BD Biosciences) were added to the cell suspension to label the living and dead cells separately. Whole transcriptome libraries were prepared using the BD Rhapsody single-cell whole-transcriptome amplification workflow. Sequencing libraries were prepared using random priming PCR of the whole-transcriptome amplification products to enrich the 3' end of the transcripts linked with the cell label and UMI. Sequencing libraries were quantified using a High Sensitivity DNA chip (Agilent) on a Bioanalyzer 2200 and the Qubit High Sensitivity DNA assay (Thermo Fisher Scientific). The library for each sample was sequenced by HiSeq X (Illumina, San Diego, CA) on a 150 bp paired-end run.

scRNA-seq data analyses for iCCA samples. For 10x Genomics scRNA-seq data analyses, raw reads were aligned to human reference genome (hg38, 2020-A) using Cell Ranger software (v6.0.0). The alignment reference and software were both provided by 10x Genomics (<https://support.10xgenomics.com>). The same software was used for unique molecular identifier (UMI) counting and filtration using default parameters. Raw count data were then analyzed with the R package Seurat (v4.0.0).

Cells that expressed less than 500 genes or over 20% mitochondrial RNA were filtered out. The expression matrix was merged and normalized using a global-scaling normalization method using Seurat. 2,000 variable genes were selected for dimensionality reduction. Principal component analysis (PCA) was performed on the variable genes and the resolution parameter to identify clusters was set to 0.8. Batch effects were corrected using ComBat. For visualization purposes, uniform manifold approximation and projection (UMAP) was performed. Clusters were identified using the top markers found in each cluster with an adjusted P value ≤ 0.05 and an average \log_2 fold change ≥ 0.1375 . Markers to identify cell populations were selected from xCell database⁴. We also used another software SingleR to verify cell populations. In the analysis of gene expression similarity between bulk RNA-seq data and scRNA-seq data, we calculated the mean expression value of scRNA-seq samples. The ssGSEA algorithm was used to calculate the enrichment score of scRNA-seq samples and bulk RNA-seq samples using IG1 and IG3 enrich genes. Z-scores were calculated from the enrichment scores.

Functional experiments

Cell lines

HuCC1, HCCC9810, HL60, and Jurkat cells are kept by Dr. Daming Gao's lab. These cell lines were cultured in RPMI.1640 medium supplemented with 10% FBS, 100 units of penicillin and 100 mg/mL streptomycin.

Plasmids and lentivirus-mediated construction of stable cell lines

The Myc-tagged coding sequence of human *KRAS* and *KRAS G12D* was cloned into the lentiviral vector pLEX-MCS-CMV-puro (Thermo Scientific Open Biosystems) to generate corresponding expression plasmids. pLEX-MCS-CMV-puro lentiviral packaging and generation of HuCCT1 (*KRAS*^{G12D} iCCA cell line) and HCCC9810 (*KRAS*^{WT} iCCA cell line) stable cell lines by infection were performed according to the protocol before¹⁷.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA was reverse transcribed into first-strand cDNA using the ABScript II RT Master Mix for qPCR Kit (ABclonal). The cDNAs were then used for real-time PCR (qPCR) on a CFX96 Touch Real-Time quantitative PCR System (Bio-Rad) using TB Green Premix® Ex Taq II (Tli RNaseH Plus; Takara). Human *GAPDH* was served as the internal control. The relative quantification of gene expression was analyzed by using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Cells were lysed in EBC lysis buffer (50 mM Tris HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40) supplemented with protease inhibitors (Selleck Chemicals) and phosphatase inhibitors (Selleck Chemicals). 30 µg total proteins were separated by 10% SDS-PAGE gel and blotted with indicated primary antibodies. Primary antibodies used for western blot analysis were as follows: Myc (2276s, Cell Signaling Technology), COX-1 (9896s, Cell Signaling Technology), COX-2 (12282s, Cell

Signaling Technology), β -actin (4970s, Cell Signaling Technology). All western blot gel images were obtained with an Minichemi 610 chemiluminescent imager (Sagecreation, Beijing, China).

Elisa

The protein levels of PGE2 and IL-2 in culture medium were quantified by enzyme-linked immunosorbent assay (ELISA) kits (KGE004B, R&D Systems and 70-EK102-48, MultiSciences). Cell-free supernatants were collected and tested according to manufacturer's protocols. Absorbance at 450 nm was measured using a microplate reader.

Transwell assay

Neutrophil model derived from the differentiation of HL60 (dHL60) cells after 120 h culture in RPIM1640 medium supplemented with 10% FBS and 1.25% dimethyl sulfoxide (DMSO). The supernatant of HuCCT1/HCCC9810 and the complete culture medium were used to prepare the conditional culture medium at the ratio of 3:1. For the neutrophil migration assay, $1 \times 10^5/200 \mu\text{l}$ neutrophils (from induced HL60 cells) were added to each upper chamber (5 μm , LABSELECT, 14331). The lower chambers contained conditioned medium from HuCCT1/HCCC9810 cells (incubated for 12 h). The number of neutrophils migrating from the upper chamber was counted microscopically.

Apoptosis detection

Jurkat T cells were pre-activated with human CD3/CD28 T cell Activator (STEMCELL, 10971) for 24 h. At the same time, dHL60 cells were treated with

indicated culture supernatants from HuCCT1 and HCCC9810. Then, the activated Jurkat T cells were cocultured with culture supernatants-treated dHL60 cells at the ratio of 1:1 for 24 hours. Cells were suspended in FACS buffer containing 1% FBS and 0.1% NaN₃ and CD3⁺ Jurkat T cells were gated for further apoptosis analysis. The data were collected on an LSR-Fortessa X20 Flow Cytometer (BD Biosciences). The annexin V/PI apoptosis flow kit was purchased from BD Biosciences (559763).

Immunohistochemistry (IHC)

Tumor tissues from mouse models were collected and fixed in 10% formalin overnight and embedded in paraffin. FFPE sections were prepared for staining using standard protocols for xylene and alcohol gradient for deparaffination. Antigen retrieval was performed in the pressure cooker (95°C for 30 min) to remove aldehyde links formed during initial fixation of tissues. Slides were incubated with primary antibodies, including anti-COX-2 (Cell Signaling Technology, 12282s), anti-Ly6G (Abcam, ab238132), anti-S100A8 (Proteintech, 15792-1-AP), anti-S100A9 (Proteintech, 14226-1-AP), anti-CD8 (Abcam, ab217344) and anti-CD19 (Abcam, ab245235). Sections were incubated and then developed with Dako REAL™ EnVision™ Detection System (DAKO, K5007). The whole IHC slide was scanned and quantified with automated acquisition system (TissueFAXS Plus, TissueGnostics GmbH, Austria).

Statistical analysis and visualization. All of the statistical analyses were performed with R software (version 4.0.2, <http://www.R-project.org>). Student's t-Test and Wilcoxon rank sum test were utilized to compare continuous and categorical variables

between two subgroups or conditions, such as immune-related score, functional-related score, clinical features and SCNA levels in each subgroup. For multi-group comparison, we used ANOVA to estimate the *P* value. Correlation matrices of immune cells were calculated using Pearson correlation and visualized using R package corrplot. Correlation plots between two features such as immune score and stromal score were also calculated using Pearson correlation. Survival curves were estimated with the Kaplan-Meier method and compared using a log-rank test. The Kaplan-Meier survival curves were plotted by R ggsurvplot package. Variables associated with overall survival were identified using univariate Cox proportional hazards regression models. Significant factors in univariate analysis were further subjected to a multivariate Cox regression analysis in a forward LR manner. The FDR correction was utilized in multiple tests to decrease false positive rates. R package ggplot2 and pheatmap were used for visualization. For functional experiments, each was repeated at least three times independently, and results were expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism (version 8).

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Supplementary Figure legends

Figure S1 Workflow and consensus clustering of multi-omics data of the FU-iCCA cohort. (A) Workflow of the iCCA multi-omics study. iCCA tumor samples from a consecutive cohort of patients were obtained for WES (n = 249), RNA-seq (n = 255), proteomics (n = 208), H&E (n = 177), multiplex immunostaining (n = 188), and scRNA-seq (n = 10) analyses. (B) ESTIMATE immune scores from our cohort and other TCGA cancer types. (C) The procedure for constructing compendium of microenvironmental cells and clustering. (D,E) Consensus cumulative distribution function (CDF) plot (D) and delta area (change in CDF area) plot (E) of 170 prognostic gene-based classification were shown. (F) Correlations between mRNA and protein abundance in 103 mRNA-protein pairs from 170 prognostic microenvironment genes. (G) Unsupervised hierarchical clustering of the FU-iCCA cohort based on the 103 proteins identified three subgroups. (H) Relative abundance and proportion of samples harboring the indicated clinical covariate in the given immune subgroup (Wilcoxon rank sum test, Pearson's chi-square test, or Fisher's exact test). (I) Representative multiplex immunostaining images to show the distribution of PDPN+ lymphatic vessels and CD31+ blood vessels among the three immune subgroups (left panel). Quantification of staining intensities for the indicated markers are shown (ANOVA, right panel). (J) Representative H&E images from the FU-iCCA cohort were shown to display the indicated pathological TIL estimates. (K) Box plot showing the pathological TILs among three immune subgroups (ANOVA). (L) Representative multiplex immunostaining images to show the distribution of

CD66b+ neutrophils among the three immune subgroups (left panel). Quantification of staining intensities for the indicated markers are shown (right panel). Green arrows: CK19-CD66b+ (ANOVA).

Figure S2 Validations of our grouping strategy in external cohorts. (A,B)

Heatmap with indicated immunogenomic features (A, left panel), Kaplan–Meier curves (A, right panel) and multivariate Cox proportional hazards model (B) for overall survival based on our subgrouping standard in Jusakul et al.’s cohort. H.R., Hazard ratio. C.I., confidence interval. (C,D) Heatmap with indicated immunogenomic features (C, left panel) and Kaplan–Meier curves (C, right panel) and multivariate Cox proportional hazards model (D) for overall survival based on our subgrouping standard in Job et al.’s cohort. H.R., Hazard ratio. C.I., confidence interval. (E-H) Comparisons of immune subgroups to the subgroups resulted from previously reported standards (E) and the heatmap of clustering analysis of Anderson et al (F), Oiishi et al (G), and Job et al (H). The *P* values were calculated by Pearson’s Chi-square-test.

Figure S3 Potential different immunogenomic features of the three immune subgroups.

(A) Principal-component feature loadings (magnitude and direction) shown in the variables factor map. Vectors were colored according to a major biological classification of cancer hallmark gene sets and ESTIMATE tumor purity. (B,C) The means of loadings per hallmark gene set for PC1 (B) and PC2 (C),

respectively. The *P* values were calculated by Wilcoxon rank sum test. (D) Hierarchically-clustered heatmap of normalized enrichment scores (NES). Rows indicated hallmark gene sets and columns indicated tumor samples. Normalized tumor purity values and immune subgroups shown on top. (E) Hallmark pathway enrichment analysis after controlling for tumor purity (adjusted ANOVA). (F-H) Comparisons of COX-IS (F), cytolytic score (G), and immunophenoscore (H) among the three immune subgroups (Wilcoxon rank sum test).

Figure S4 Association of the immune subgroups with genomic alterations. (A) Comparison of total SCNA levels among the three immune subgroups (Wilcoxon rank sum test). (B) Correlation analysis between ESTIMATE immune score and SCNA burden. (C) The expression heatmaps depicted mRNA and protein levels of various markers involved in cancer-promoting or cancer-inhibitory inflammation (Wilcoxon rank sum test). (D,E) Comparisons of COX-IS (D) and cytolytic score (E) in tumor samples with and without *KRAS* mutation (Wilcoxon rank sum test). (F) GSEA analysis based on the MDSC signature up gene set in tumor samples with and without *KRAS* mutation. (G-I) Comparisons of neutrophil infiltration (G), COX-2 mRNA level (H), and S100A8 mRNA level (I) of patients with and without *KRAS* mutations in Jusakul's cohort (Wilcoxon rank sum test). (J) Pathway enrichment analysis based on differentially expressed genes that associated with *KRAS* mutations in Jusakul's cohort (Wilcoxon rank sum test). (K-O) HuCCT1 and HCCC9810 cells ectopically expressing Myc-tag *KRAS*^{WT} or Myc-tag *KRAS*^{G12D} were generated and then

qRT-PCR (K,L), western blot (M), and ELISA (O) were performed to detect the indicated molecules. Quantification of three independent western blotting assays were shown (N). The NC group indicated cell lines without transfection. Representative data of triplicate experiments were shown. Data are expressed as mean \pm SEM (ANOVA). nd, not determined. (P) Transwell assay showing that the culture supernatants from KRAS^{WT} and KRAS^{G12D} overexpressing HuCCT1 and HCCC9810 significantly enhanced the migration of dHL60. Representative data of triplicate experiments were shown. Data are expressed as mean \pm SEM (ANOVA). (Q) Culture supernatants from KRAS^{G12D} overexpressing HuCCT1 and HCCC9810 treated with DMSO or 15 μ M COX-2 inhibitor Celecoxib for 48 h were collected. Then the DMSO treated supernatant (DMSO group), DMSO treated supernatant plus 15 μ M Celecoxib (CELE untreated group), and Celecoxib treated supernatant (CELE treated group) were used to perform the transwell assay. Representative data of triplicate experiments were shown. Data are expressed as mean \pm SEM (ANOVA). (R,S) The effects of pre-treatment dHL60 by indicated culture supernatants on anti-CD3/CD28 activated Jurkat T cells. The apoptosis (R) and IL-2 (S) secretion of Jurkat T cells were detected. Representative data of triplicate experiments were shown. Data are expressed as mean \pm SEM (ANOVA).

Figure S5 The KRAS mutation-associated tumor microenvironment of mouse iCCA model. (A) Schematic of vectors used in the hydrodynamic injections. Luc, luciferase. (B,C) Immunohistochemical analysis for CD8 and CD19 (B) and

quantification of staining intensities for the indicated markers are shown (C). Representative data of triplicate experiments were shown. Data are expressed as mean \pm SEM. (D) UMAP plot showing the annotation and color codes for cell types in the FU-iCCA ecosystem. (E) Violin plot showing the expression of marker genes in the indicated cell types. The top dots label the clusters corresponding to specific cell types in (D) and the bottom shows indicated marker genes. (F) UMAP plot showing the group origin in AY and AYK mouse iCCA samples. (G) Violin plots showing *Ptgs2* and *Il1b* expression of indicated cell composition in AY and AYK samples, respectively. The *P* values were calculated by ANOVA. (H) Overview of *KRAS* mutation-dominated myeloid inflammation.

Figure S6 Immunogenomic features of IG2. (A) Comparison of TNB among the three immune subgroups (Wilcoxon rank sum test). (B-D) Comparison of TCR diversity (B), BCR diversity (C), and RNA-seq reads mapping to VDJ loci (D) among the three immune subgroups (Wilcoxon rank sum test). (E) Heatmap showed the relative distribution of co-inhibitors (left panel) and co-stimulators (right panel) among the three immune subgroups (adjusted ANOVA). (F) Associations of HLA LOH with immune subgroups, clinicopathologic factors, and multi-omics profiles (Pearson's Chi-square-test, Fisher's exact test, or ANOVA).

Figure S7 Multi-omics features related to TLSs. (A) Associations of intra-tumoral TLSs with TLS score, immune subgroups, clinicopathologic factors, and multi-omics

profiles (ANOVA, Pearson's chi-square test, or Fisher's exact test). (B) Comparisons of xCELL enrichment scores of indicated immune subsets between TLSs- and TLSs+ subgroups (ANOVA). (C) Comparisons of calculated scores of co-inhibitors or co-stimulators between TLSs- and TLSs+ subgroups (ANOVA). (D,E) Comparison of cytolytic scores (D) and COX-IS (E) between TLSs- and TLSs+ subgroups (Wilcoxon rank sum test).

Figure S8 Influences of HBV infection on the immune microenvironment of iCCA. (A) Comparisons of HBV infection in patients of IG1 and IG2/IG3 (Fisher's exact test). (B) UMAP plot, showing the annotation and color codes for cell types in the FU-iCCA ecosystem. (C) PCA analysis of all FU-iCCA samples based on the genes exclusively enriched in IG1 and IG3. The 10 scRNA-seq samples were also projected onto the plot. (D) Dot plot of cell types assignment for indicated genes in scRNA-seq. Dot size indicated fractions of expressing cells, colored according to z-score normalized expression levels. (E,F) Bubble heatmap showing marker genes across T/NK subgroups (E) and myeloid subgroups (F) from tumor samples. Dot size indicated fraction of expressing cells, colored according to z-score normalized expression levels. (G) Immune clusters showing significant differences in the comparison among the three immune subgroups. The color saturation represented the ration to T/NK or myeloid subgroups. The *P* values were calculated by ANOVA. (H-J) Volcano plot showed differently expressed genes of CD4+ T cells (H), NK cells (I), and macrophages (J) in HBV positive versus HBV negative tumor samples.

